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Effect of Zinc Ions on the Biochemical Behavior of Simian Virus 40 Small-t Antigen Expressed in Bacteria

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The simian virus 40 small-t antigen contains 10 cysteine residues, 6 of which are organized in two CysXCysXXCys clusters. Mutation of individual Cys residues in the two clusters or mutation of specific residues found between these clusters causes pronounced instability of the protein in animal cells. Protein instability correlates with failure of the bacterially expressed mutant proteins to bind zinc ions, an interaction which allows purification of large amounts of small-t antigen in monomeric form.

The simian virus 40 (SV40) small-t antigen (small-t) is a 17-kDa polypeptide which shares 82 N-terminal amino acids with the large-T antigen and contains a unique C-terminal domain of 92 amino acids (18). The unique C-terminal domain is cysteine rich, with two characteristic clusters (CysXCysXXCys) that are found in the small-t antigens of all papovaviruses and in the polyomavirus middle-T antigen (6). In a recent study, we showed that mutation of any one of the Cys residues in the two clusters made small-t antigen highly unstable in animal cells (7). All other mutations described in this earlier study made small-t antigens with stabilities similar to that of the wild-type (WT) protein.

While mutational analyses were under way, efforts to purify large amounts of monomeric small-t antigen from bacteria were also in progress. As described previously (3, 17), aggregation of small-t has been a consistent problem in purification of small-t from bacteria, and inclusion of reducing agents such as dithiothreitol (DTT) was not sufficient to maintain small-t in monomeric form. Purified small-t antigen was needed to study the interaction of this viral protein with cellular 61- and 37-kDa proteins, now known to be regulatory (A) and catalytic (C) subunits of protein phosphatase 2A (PP2A) (12, 19). Like cellular B subunits of PP2A, small-t binds PP2A through the regulatory A subunit (8). It has been important to isolate monomeric small-t because aggregated forms of small-t were found to be associated with large numbers of bacterial proteins and to interact nonspecifically with added cellular proteins (3). In contrast, the small amounts of monomeric small-t which could be isolated were able to recognize radiolabeled 61- and 37-kDa proteins from extracts of uninfected monkey cells.

In this report, we show that the behavior of small-t antigen expressed in bacteria is influenced by the ability of the protein to bind zinc ions and that inclusion of zinc ions in purification of small-t allows isolation of large amounts of monomeric protein. Mutations of individual Cys residues of the two Cys clusters, which lead to instability of small-t antigen in animal cells (7), also result in the inability of the protein to be recovered in monomeric form. In addition, two mutations in the sequence between the two Cys clusters result in both instability in infected cells and aggregation of bacterially expressed protein.

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MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (CV1) cells were grown in Dulbecco modified Eagle medium containing 5% fetal bovine serum. All virus stocks were harvested from lysates initiated with a low multiplicity of virus, and titers were determined on CV1 cells. Viruses with mutant small-t antigens were described previously (7), and they grew to titers at least one-fifth that of WT SV40. Equal multiplicities of infection were used in all experiments.

Plasmids with WT and mutant small-t antigens. Mutant small-t antigens were produced in plasmid pw2 or pw2t as described previously (4, 7, 14). Mutant X23 was produced by cleavage of pw2 at the unique SV40 *Bst*XI site (nucleotide [nt] 4759), treatment with T4 3′-exonuclease, and then ligation of a 10-nt linker containing a *Cla*I site, 5′-CCATC GATGG-3′ (New England Biolabs). Following *Cla*I digestion and ligation, the resultant plasmid was used to isolate the SV40 early region (*Kpn*I-*Bam*HI digestion), which was ligated to the SV40 late region and transfected into CV1 cells with DEAE-dextran. Isolated viral DNA was sequenced to confirm the predicted sequence. The mutations of His-122 to Leu, of Cys-97 to Ser, and of Cys-103 to Ser were produced from mutant oligonucleotides by polymerase chain reaction with a Cetus thermocycler. Following amplification, restriction fragments were cloned into pw2t and resultant plasmids were sequenced to show the presence of the desired mutations and to show that there were no others.

For expression of mutant small-t antigens in bacteria, the plasmid pKK223-3 was used (Pharmacia). Expression of a 14-kDa derivative of small-t occurs from an internal ribosome binding site in these constructs that uses the ATG codon at nt 5008. Plasmids were constructed by cloning an SV40 *Hin*dIII fragment (nt 5171 to 4002) into the *Hin*dIII site of pKK223-3 or by cloning an SV40 *Stu*I-*Hin*dIII fragment (nt 5190 to 4002) into the *Sma*I and *Hin*dIII sites of pKK223-3. Equivalent levels of expression of the 14-kDa small-t antigen were observed in either construction. Some mutations were also cloned in the Pharmacia vector pTrc99A, from which the 17-kDa small-t antigen could be expressed. The ATG codon of this vector falls within an *Nco*I site. Consequently, mutant sequences were amplified by polymerase chain reaction with a forward primer that contained the *Nco*I site (5′-GCCATGGATAAAGTTTTA AACAG-3′) as well as a reverse primer from SV40 nt 3950. Following amplification, an *Nco*I-*Hin*dIII (nt 4002) fragment was cloned into pTrc99A. Constructs were sequenced

through the small-t coding region to ensure that only desired mutations were present.

Purification of small-t from bacteria. Overnight cultures (2 ml) of pTR865 (2) or bacteria which contained the pKK223-3 or pTrc99A derivatives were inoculated into 100 ml of M9 medium containing ampicillin and were grown at 37°C for 2 h. Isopropyl-β-D-thiogalactopyranoside (IPTG) (5 mM) and, when indicated, [^{35}S]methionine were then added for 2 h. Bacteria were collected by centrifugation and digested with lysozyme in the presence of EDTA, and then spheroplasts were collected by centrifugation. Spheroplasts were lysed in buffer containing 20 mM Tris-Cl (pH 8), 80 mM NaCl, 50 mM $MgCl_2$, 0.1% Triton X-100, and 1 μg of DNase per ml at room temperature until viscosity was reduced. The bacterial debris fraction was then collected by centrifugation in a microcentrifuge, washed once, and suspended in 10 M urea in 20 mM Tris-Cl (pH 8), 80 mM NaCl, 2 mM DTT, and 0.25 mM $ZnSO_4$ for 1 h at room temperature. Soluble proteins were recovered following centrifugation in a microcentrifuge. For large-scale purification, the urea-soluble protein fraction was diluted twofold with 20 mM Tris-Cl (pH 8), 80 mM NaCl, 2 mM DTT, and 0.02% Triton X-100 and then pumped onto an upward flow G200 column (2.5 by 40 cm) equilibrated in the same buffer. Fractions (5 ml) were collected over a 16-h period. Fractions containing monomeric small-t were passed through a column (2 by 4 cm) of DEAE-cellulose. Proteins which did not bind DEAE were then applied to a column (2 by 3 cm) of hydroxylapatite (Ultrogel). After extensive washing, small-t was eluted from the hydroxylapatite with 100 mM potassium phosphate (pH 7.6), 80 mM NaCl, 2 mM DTT, and 0.02% Triton X-100. Triton X-100 was found to be essential for recovery of small-t, which cannot be eluted from hydroxylapatite in the absence of detergent. Concentrated proteins were dialyzed overnight against buffers needed for later experiments, and small-t was found to maintain activity in a variety of salt concentrations and pH conditions.

Blotting for zinc binding. Binding of ^{65}Zn by proteins immobilized on nitrocellulose was done as described by others (1, 16). Briefly, 5 μg of small-t or each protein standard was separated on a 15% sodium dodecyl sulfate (SDS) gel, and then the proteins were transferred to nitrocellulose. After transfer, the proteins were incubated in renaturing buffer (100 mM Tris-Cl [pH 6.8], 50 mM NaCl, 10 mM DTT) for 1 h. Filters were washed briefly with N_2-flushed buffer without DTT and then incubated with 50 to 100 μCi of $^{65}ZnCl_2$ in this buffer for 30 min. Filters were then washed in a buffer like that described above but containing 1 mM DTT.

Immunoprecipitation. Proteins expressed by infected CV1 cells were extracted and immunoprecipitated as described previously (7) by using polyclonal sera from tumor-bearing hamsters. Proteins translated in vitro were immunoprecipitated with polyclonal hamster sera.

RESULTS

A mutation between the cysteine clusters affects stability in CV1 cells. We showed previously that small-t antigen from infected monkey kidney cells had a half-life of over 6 h and that mutation of Cys residues in the two CysXCysXXCys clusters (Cys residues 111, 113, 116, 138, 140, and 143) led to severe instability of the small-t antigen. The mutant proteins had half-lives of 30 to 60 min (7). Recently, two mutations which also result in labile small-t antigens even though no Cys residue is mutant have been obtained. The first mutation, X23, consists of an insertion at amino acid 123. This mutation converts the WT sequence of small-t from Pro-Leu-Val-Trp to Pro-Pro-Ile-Asp-Gly-Trp, increasing the distance between the two cysteine clusters to 23 amino acids. The second mutation alters the His-122 residue to Leu. As shown in Fig. 1, levels of small-t antigen expressed by the His-122 mutant virus were equivalent to those of WT virus in short (1-h) pulses (lanes a and b), but the mutant small-t antigen was rapidly degraded (lanes d, f, and h). The WT protein was stable during the chase periods (lanes c, e, and g). Similar instability was noted for X23.

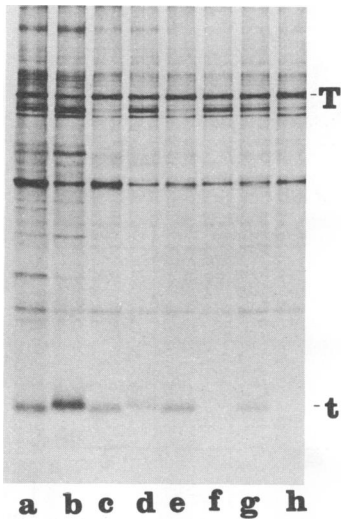

FIG. 1. Instability of the His-122 mutant small-t antigen. Equal multiplicities of WT SV40 (lanes a, c, e, and g) and the His-122 mutant virus (lanes b, d, f, and h) were used to infect replicate cultures of CV1 cells in 35-mm-diameter dishes. After 40 h, all plates were pulsed with [^{35}S]methionine in methionine-free medium for 1 h, one set was extracted (lanes a and b), and then three plates were washed and incubated with complete medium for 1 h (lanes c and d), 2 h (lanes e and f), and 3 h (lanes g and h). Extracts were used for immunoprecipitation with polyclonal hamster sera, and the immunoprecipitated proteins were separated on SDS gels as described previously (7). T, large-T antigen; small-t antigen.

Effect of Zn on purification of small-t. The instability of mutations such as X23, His-122 to Leu, and those in the Cys clusters suggested the possibility that the structure of the unique C-terminal half of small-t antigen was being disrupted by the mutations. It seemed possible that either intracellular disulfide bond formation or binding to heavy metals might be responsible for the behavior of the mutant proteins. A role for zinc ions was first suggested in efforts to purify small-t antigen. Inclusion of zinc has a dramatic effect on recovery of monomeric small-t antigen from bacterial clones which express this protein. Small-t was purified from bacteria which contained plasmid pTR865 (2), in which small-t is expressed from the *tac* promoter. In addition to the 17-kDa small-t antigen, a 14-kDa truncated form of small-t is expressed from an internal translation initiation site used in bacteria. Following induction of bacterial cells with IPTG, the small-t antigen produced by pTR865 was found in insoluble fractions but could be recovered by extraction with 10 M urea. When labeled with [^{35}S]methionine, 30 to 50% of the radioactivity incorporated during the induction phase was found to be in the insoluble cell material, whereas in control bacteria, only 3 to 5% of the radioactivity was found in this fraction.

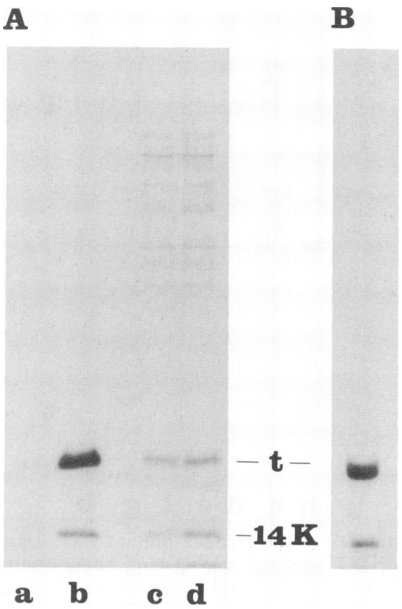

FIG. 2. Effect of zinc on DEAE-cellulose elution of small-t. (A) Parallel 10-ml cultures of pTR865 were grown and induced as described in Materials and Methods. Small-t (t) was solubilized from one culture in buffer containing 2 mM DTT but no zinc ions (lanes a and c). Small-t was solubilized from the second culture in the presence of 0.25 mM $ZnSO_4$ (lanes b and d). The solubilized proteins were then diluted 15-fold with buffer containing 2 mM DTT and applied to DEAE-cellulose columns (1 by 3 cm). Proteins which did not bind DEAE are shown in lanes a and b, and those eluted from DEAE with 400 mM NaCl are shown in lanes c and d. Patterns shown are on SDS gels stained with Coomassie blue, and they were identical to patterns obtained by autoradiography of radioactive proteins. (B) Protein purified as described in Materials and Methods by G200, DEAE-cellulose, and hydroxylapatite chromatography.

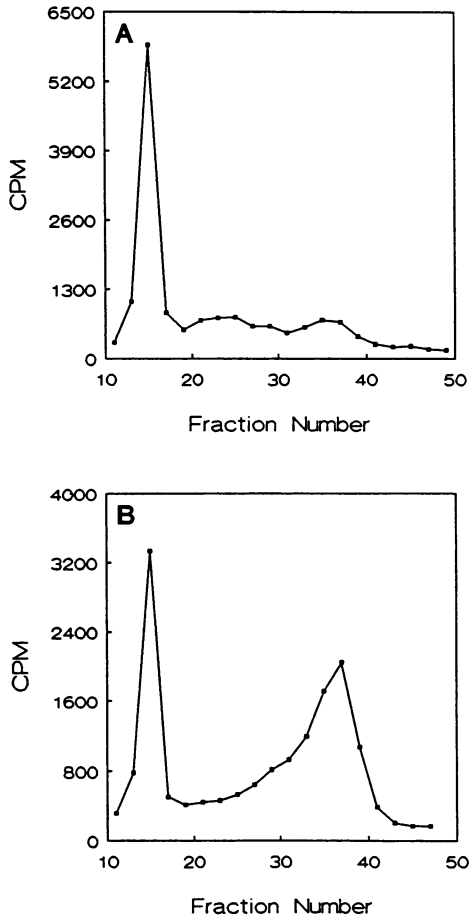

FIG. 3. G200 chromatography of small-t antigen. Two 50-ml cultures were induced and extracted as described for the DEAE-cellulose experiment, but cellular debris pellets were frozen at −80°C before use. For the first column (A), one pellet was thawed and then incubated with 3 ml of urea-containing buffer without zinc. After insoluble material was removed, the soluble proteins were fractionated on a G200 column (2.5 by 40 cm). The second pellet was then solubilized with 0.25 mM $ZnSO_4$ present and pumped onto the G200 column (B). About 40% of the radioactivity was recovered in fractions 35 to 45, the region of the column where a marker protein, myoglobin (17 kDa), migrated.

We have shown previously that monomeric small-t failed to bind DEAE-cellulose at pH 8 in the presence of 80 mM NaCl, whereas aggregated forms of small-t were largely retained by this column (3). The first indication that zinc might influence the state of small-t antigen came from these columns. As shown in the stained gel in Fig. 2, inclusion of zinc in the solubilization buffer resulted in greatly increased levels of small-t antigen in fractions which did not bind DEAE-cellulose (lane b), compared with proteins solubilized in the absence of zinc (lane a). The majority of contaminating cellular proteins bound DEAE (Fig. 2, lanes c and d) and could be separated from small-t antigen. Less total small-t was recovered from these preparations in the absence of zinc because highly aggregated small-t molecules were not recovered from the DEAE columns.

The monomeric nature of part of the small-t antigen solubilized in the presence of zinc was shown by G200 chromatography. Small-t extracted in the absence of zinc was largely aggregated and was found primarily in the void fractions following chromatography (Fig. 3A). Inclusion of zinc in the solubilization step resulted in a pronounced peak of radioactivity in the monomeric region of the column (fractions 30 to 40) (Fig. 3B). The elution profile of a 17-kDa marker protein, myoglobin, coincided with the second peak of radioactivity shown in Fig. 3B. Protein present in this region was almost exclusively small-t antigen and the 14-kDa protein when analyzed by staining SDS gels.

Extensive purification of the small-t and 14-kDa protein mixture can be accomplished by combining G200 chromatography, DEAE-cellulose, and hydroxylapatite chromatography as described in Materials and Methods. Protein recovered in these procedures is over 90% pure (Fig. 2B). Similar preparations of purified small-t antigen have been used in studies of PP2A activity and shown to inhibit this cellular enzyme (15, 20).

Direct binding of zinc by small-t antigen. The behavior of small-t in purification suggested that the protein bound zinc ions. One indication that this is the case came from zinc-blotting experiments (Fig. 4). Small-t antigen and several control proteins were separated from other proteins on SDS gels and then transferred to nitrocellulose. The nitrocellulose sheets were then reduced in the presence of DTT and incubated with $^{65}ZnCl_2$. Following this procedure, it was found that both the 17-kDa small-t antigen and the 14-kDa truncated small-t produced in bacteria were able to bind

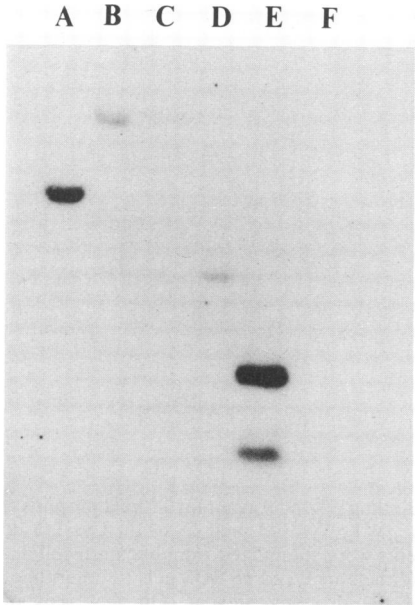

FIG. 4. Binding of zinc by small-t transferred to nitrocellulose. A total of 5 μg of both purified small-t and the 14-kDa protein was separated on a 15% SDS gel along with 5 μg each of five control proteins. Following transfer to nitrocellulose, the blots were reduced and then incubated with 50 μCi of $^{65}ZnCl_2$ as described in Materials and Methods. As previously shown (1, 16), alcohol dehydrogenase, alkaline phosphatase, and carbonic anhydrase all bind zinc, although the latter two proteins show less binding when reduced. Bovine serum albumin, a cysteine-rich protein, and lysozyme did not bind zinc. Lanes: A, alcohol dehydrogenase; B, alkaline phosphatase; C, bovine serum albumin; D, carbonic anhydrase; E, small-t and the 14-kDa protein; F, lysozyme.

zinc. Bovine serum albumin and lysozyme, both Cys-rich proteins, are important negative controls in this experiment and show that not all Cys-rich proteins bind zinc in this assay.

Although data are not shown here, binding of ^{65}Zn by small-t antigen was also shown by immunoprecipitation of small-t translated in vitro from transcripts generated with T7 polymerase.

Effect of Cys mutations on biochemical behavior. To demonstrate that instability of a mutant small-t protein correlated with its inability to bind zinc, WT and Cys-143 mutant sequences were cloned individually into the vector pKK223-3 (Pharmacia), which contains the *tac* promoter followed by polylinker cloning sites. In these constructions, the initiating ATG of small-t is too far removed from a ribosome binding site to be translated efficiently in bacteria. However, expression of the 14-kDa derivative from an internal ATG occurs with the same efficiency as in pTR865, and the 14-kDa protein binds zinc. As shown in Fig. 5B, the Cys-143 mutation which caused instability in animal cells resulted in the failure of Zn to allow recovery of the protein in monomeric form.

Our experience to date suggests that the 14-kDa protein is an active form of small-t antigen. Not only does this protein bind zinc (Fig. 4), but the 14-kDa protein can be purified by using the approaches described for small-t. Most importantly, the 14-kDa truncated protein inhibits PP2A activity as efficiently as the full-length protein (10a). However, to confirm that the biochemical behavior of the 14-kDa protein

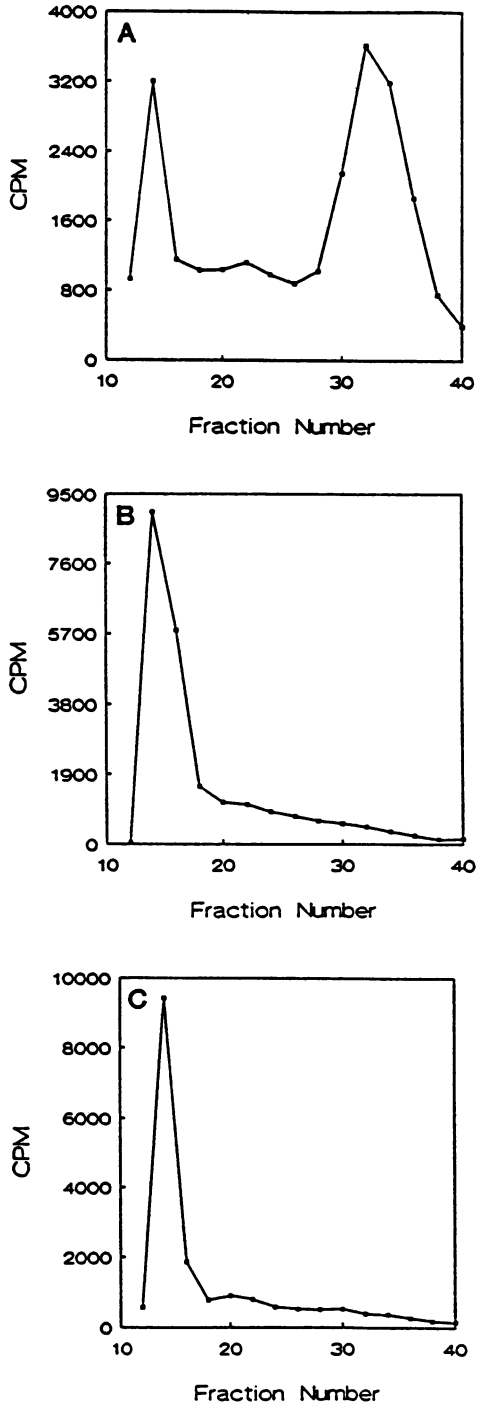

FIG. 5. G200 chromatography of the WT and Cys-143 mutant 14-kDa and 17-kDa proteins. WT and Cys-143 mutant small-t sequences were cloned into either the pKK223-3 or the pTrc99A plasmid as described in Materials and Methods. Log-phase cultures were induced and extracted, and then small-t antigens were solubilized in the presence of zinc ions from cell debris fractions. Patterns shown are the G200 profiles obtained from the WT 14-kDa protein (A) and the Cys-143 mutant 14-kDa protein (B) expressed from pKK223-3 and the Cys-143 17-kDa protein expressed from pTrc99A (C).

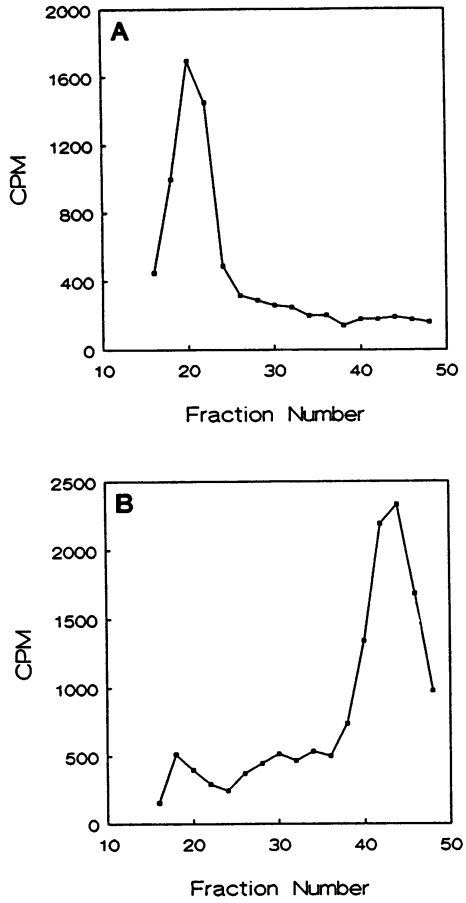

FIG. 6. G200 chromatography of Cys-97 and His-122 mutant 14-kDa proteins. Induction and extraction of cells carrying mutations cloned into pKK223-3 were as described in the legend to Fig. 5. (A) His-122; (B) Cys-97.

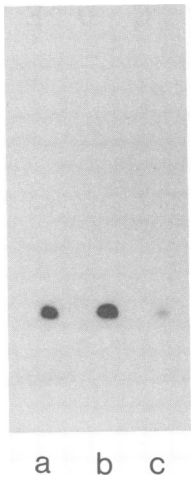

FIG. 7. Binding of zinc by mutant 14-kDa proteins transferred to nitrocellulose. WT, Cys-143, and Cys-116, Cys-143 double-mutant 14-kDa proteins were solubilized from bacterial cell debris fractions with urea and then analyzed on SDS gels to determine approximate quantities of the proteins present. On the basis of Coomassie blue-stained patterns, equivalent quantities of each protein were loaded on a second SDS gel, transferred to nitrocellulose, reduced, and incubated with $^{65}ZnCl_2$ as described in Materials and Methods. (a) Cys-143; (b) WT; (c) Cys-116, Cys-143.

accurately reflected the effect of mutations on the full-length protein, the Cys-143 mutation was cloned into the pTrc99A expression vector following introduction of an *Nco*I site at the initiating ATG during amplification. This construct expressed both the 17- and the 14-kDa forms of small-t as the original pTR865 did. As in the case of the 14-kDa protein, the mutant small-t antigen expressed from pTrc99A failed to renature to monomeric form following urea solubilization in the presence of zinc (Fig. 5C). Similar results were obtained with the Cys-138 and Cys-140 mutations, while WT small-t was recovered in monomeric form when expressed from the pTrc99A vector (data not shown).

Mutations outside the Cys clusters. Additional mutant proteins have also been expressed in bacteria, and their behavior on G200 chromatography has been monitored. For example, mutation of His-122 to Leu resulted in aggregation of the 14-kDa protein after renaturation in the presence of zinc, such that all protein was found in the void volume of G200 columns (Fig. 6A). A similar pattern was obtained when the 17-kDa form of this mutation was expressed from pTrc99A (data not shown). This suggests that the instability of the His-122 mutant protein observed in animal cells (Fig. 1) may result from its inability to bind zinc, as is the case for the mutations of Cys residues in the two Cys clusters. Not all mutations in the region between the two Cys clusters have this effect. For example, monomeric 14-kDa protein was readily obtained from a mutation in which Val-134 was replaced by Met (data not shown). Two additional mutations have also been of interest. These altered either Cys-97 or Cys-103 to Ser. These Cys residues are highly conserved in papovavirus small-t antigens but are located upstream of the two Cys clusters. These mutations resulted in 14-kDa small-t antigens which could be recovered in monomeric form following renaturation in the presence of Zn ions (Fig. 6B), additional evidence that not all Cys residues in small-t influence the stability of the protein. We had shown previously that mutations at Cys-153 and Cys-161 did not cause small-t antigen to become unstable in CV1 cells (7).

Binding of zinc by immobilized mutant proteins. Surprisingly, when the 14-kDa Cys-143 mutant protein expressed in bacteria was analyzed by the Zn blotting procedure, reduced but detectable zinc binding was observed (Fig. 7), even though the single mutation completely eliminated the ability of the protein to maintain its monomeric state in G200 chromatography. Presumably, remaining Cys residues of the two clusters retain the ability to bind zinc at least weakly. To further explore this finding, a double mutation (Cys-116,Cys-143) was constructed in the pKK223-3 plasmid and expressed in bacteria. The double-mutant protein showed even further reduction in zinc binding, but positive signals were still obtained with the double-mutant protein, compared with control proteins such as lysozyme, which failed to bind zinc on this blot. Similar results were obtained by immunoprecipitation of ^{65}Zn with small-t translated in vitro. Mutation of additional Cys residues in the two clusters may be required to completely eliminate zinc binding.

DISCUSSION

The pronounced instability of several small-t mutant proteins, notably those which affected Cys in the two CysX

CysXXCys clusters, suggested that the Cys clusters were involved in the structural integrity of the protein. The results presented here suggest that the role of this region of small-t may be to bind heavy metal ions such as zinc. Inclusion of zinc during solubilization of small-t expressed in bacteria had pronounced effects on the recovery of monomeric small-t protein, allowing purification of the viral protein for studies of its effects on PP2A (15, 20). It remains to be determined whether isolation of small-t in monomeric form from other sources such as baculovirus (11) would be enhanced by inclusion of zinc ions during isolation of the protein.

The association of zinc with bacterially expressed small-t antigen is very stable, and zinc remains with the purified protein even though the ion is not included in any buffers after the initial solubilization. This has been shown by atomic absorption spectroscopy (18a). Zinc is not released from small-t or the 14-kDa derivative by passage through Chelex 100, a metal-chelating resin, or by treatment with EDTA.

Several proteins which use Cys residues to coordinate zinc binding are known. Most studies of zinc-binding proteins have involved nuclear transcription factors, e.g., TFIIIA (10) and Gal4 (13). TFIIIA and the SV40 large-T antigen (9) have the commonly found zinc finger structure Cys-X(2–4)-Cys– – –His-X(2–4)-His, which is believed to bind a single zinc ion. The zinc binding region of Gal4 more nearly resembles that found in small-t antigen:

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Cys-X2-Cys-X6-Cys-X6-Xys-X2-Cys-X6-Cys (Gal4)
Cys-X--Cys-X2-Cys-X21-Cys-X-Cys-X2-Cys (Small-t)
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The Gal4 protein is unique in that six Cys residues coordinate the binding of two zinc ions (13). Preliminary analyses suggest that small-t will also bind more than one zinc ion as well (18a).

Binding of more than one zinc ion per molecule of small-t may partially explain the behavior of mutant proteins in zinc-blotting assays, because the binding of a single zinc ion can be coordinated by four Cys residues, as observed for proteins such as the steroid receptors (5). Mutation of the correct pair of Cys residues, or mutation of three of the six residues, may be required to completely eliminate zinc binding in the blotting assay. The important observation, however, is that mutation of only a single Cys residue is sufficient to alter the biochemical behavior of small-t, either in vivo or in purification of bacterially expressed products. Other amino acid residues in small-t also play roles in protein stability and zinc binding, as indicated by the mutation of His-122 and the X23 insertion.

It should be noted that we have not yet shown that small-t isolated from animal cells is found in association with zinc or another heavy metal ion. Small-t clearly has the capacity to bind zinc ions, but this may not be the state of the protein intracellularly. Other interactions, such as disulfide bond formation between the Cys residues, could explain the instability of the mutant proteins in animals cells, and structural stabilization of the protein through disulfide bonds remains a formal possibility.

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